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(71) Applicant *(for all designated States except US)*: RSR LIMITED [GB/GB]; Avenue Park, Pentwyn, Cardiff CF2 7HE (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: GRENNAN JONES, Fiona [IE/GB]; 126 Pontygwindy Road, Caerphilly CF83 3HF (GB). FURMANIAK, Jadwiga [GB/GB]; 35 Havenwood Drive, Thornhill, Cardiff CF4 9HX (GB). REES SMITH, Bernard [GB/GB]; Richmond House, Druidstone Road, Old St. Mellons, Cardiff CF3 9BX (GB).

(74) Agent: AUSTIN, Hedley, William; Urquhart-Dykes & Lord, Alexandra House, Alexandra Road, Swansea, West Glamorgan SA1 5ED (GB).

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(54) Title: MONITORING REACTIVITY OF THYROID PEROXIDASE AUTOANTIBODIES

(57) Abstract

A method of monitoring the reactivity of thyroid peroxidase (TPO) autoantibodies (Abs) in order to distinguish between disease-related and unrelated TPO Abs which method comprises: (a) labelling modified TPO prepared using expressed TPO genes containing truncations and/or deletions; and (b) monitoring reactivity of the labelled modified TPO with TPO autoantibodies present in a body fluid from a patient.

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Monitoring Reactivity of Thyroid Peroxidase Autoantibodies

The present invention relates to a method of monitoring the reactivity of thyroid peroxidase (TPO) autoantibodies (Abs) in order to distinguish between disease-related and disease-unrelated TPO Abs.

Almost 40 years ago the reactivity of sera from patients with Hashimoto's thyroiditis, a thyroid specific antigen distinct from thyroglobulin, was described (Belyavin & Trotter; The Lancet; pp648-652; 1959). This antigen had been shown to be localised in the cytoplasmic fraction of thyroid follicular cells and was referred to as "thyroid microsomal antigen". Further studies have shown clearly that the microsomal antigen is thyroid peroxidase (TPO) -the enzyme involved in iodination of tyrosines in thyroglobulin i.e. formation of thyroid hormones.

Methods to measure Abs to the thyroid microsomal antigen were traditionally based on partially purified antigen preparations used in complement-fixing assays, immunofluorescence tests or systems based on antigen-coated cells or particles (e.g. tanned red cell/haemagglutination techniques), antigen-coated enzyme linked immunosorbent assay (ELISA) plates or antigen-coated tubes.

After TPO had been identified as the major component of thyroid microsomal antigen, assays to measure TPO antibodies were more commonly based on purified preparations of native or recombinant TPO. The currently used methods include ELISA and radioimmunoassay (RIA) techniques.

TPO autoantibodies are known to be markers of autoimmune thyroid disease (AITD) such as Graves' disease and Hashimoto's thyroiditis. Approx 80% of patients with AITD are found to have TPO Abs and the levels of TPO Abs in these patients are usually very high. However, TPO Abs, albeit at lower levels, are found in approx 20% of healthy female blood donors. The prevalence of TPO Abs increases with age, rising from approx 15% in the 18 to 24 age group, to approx 25% in the 55 to 64 age group for healthy females. The prevalence of TPO Abs in healthy male blood donors is approx 10%.

In the cases of Abs to thyroglobulin (Tg - another thyroid specific autoantigen) it has been shown that Tg Abs from healthy blood donors and Tg Abs from patients with AITD appear to be directed to different epitopes on the Tg molecule. There has been evidence that autoantibody epitopes on human TPO as well as those on Tg are conformational However, it is also believed that two short C-terminal sections of TPO amino acids ((AA) 590-621 = C2 and AA 709-721 = C21) can bind TPO Abs.

We have now found that it is possible to distinguish between disease-related TPO Abs due to differences in reactivity of TPO Abs from healthy blood donors and TPO Abs from patients with AITD or other autoimmune diseases.

According to the present invention there is provided a method of monitoring the reactivity of TPO Abs, which method comprises:

(a) labelling modified TPO prepared using expressed TPO genes containing truncations and/or deletions; and

(b) monitoring reactivity of the labelled modified TPO with TPO autoantibodies present in body fluid from a patient (using, for example, an immunoprecipitation assay).

The modified TPO itself therefore contains corresponding truncations and/or deletions.

In experiments leading to the present invention full-length human TPO cDNA (coding for a 933AA protein) was digested at the AccI site to yield a 90kD (838AA) extracellular, water-soluble fragment. Previous studies have shown that the reactivity of TPO Abs to the 90kD fragment and the full length TPO (116kD) was similar. Consequently, the 90kD fragment was used as a reference for analysis of TPO autoantibody binding.

The TPO 90kD reference preparation and the modified TPO preparations (including those with the N-terminal amino acids 3-166 and/or 3-324 deleted) are used. The TPO preparations (reference and modified) can be labelled with different reagents (radiologically, for example with ^{125}I or ^{35}S methionine) or chemically, for example with chemiluminescent reagents, bioluminescent reagents, fluorescent compounds, enzymes, metal chelates or a dye (such as a streptavidin dye complex). The difference in the ability of TPO Abs to react with reference TPO and modified TPO will indicate the presence of disease-related TPO Abs or disease-unrelated TPO Abs.

It is thereby possible to distinguish between disease-related TPO Abs and disease-unrelated TPO Abs.

In one embodiment the autoantibodies are monitored using an ELISA. In the ELISA, diluted serum samples are added to TPO coated plastic wells of a standard ELISA plate and incubated to allow for TPO autoantibody binding to TPO on the well wall to occur. Bound autoantibody is then quantified typically by addition of anti-human IgG (or protein A) coupled to an enzyme such as horseradish peroxidase or alkaline phosphatase, addition of an appropriate enzyme substrate which forms a coloured reaction product and quantification of the colour intensity using a spectrophotometer. The colour intensity is a function of the amount of Abs in the serum sample. A standard curve can be included in the assay to give fully quantitative results, or results can be expressed as an assay index in which the colour intensity (optical density or OD) in the test sample is divided by the OD observed for a standard reference TPO autoantibody preparation.

Another method which may be used for monitoring TPO Abs is a radioimmunoassay (RIA). Several RIA systems are suitable for monitoring TPO Abs; in the majority of cases these assays use reagents radioactively labelled with ^{125}I. Some of these assays employ sandwich-type systems, in which TPO Abs in a test serum bind to TPO coated tubes and are then detected by addition of either ^{125}I-labelled TPO, ^{125}I-labelled anti-human IgG or ^{125}I-labelled protein A. Alternatively in an inhibition assay, TPO monoclonal antibody can be coated onto the plastics tubes and the tubes will bind ^{125}I-labelled TPO. TPO Abs in test sera will inhibit the binding of ^{125}I-TPO and results can be read off a standard curve. Highly sensitive TPO autoantibody assays depend on the direct liquid phase interaction between TPO Abs and ^{125}I-labelled antigen. In this type of assay, diluted serum samples are first incubated with ^{125}I-labelled TPO to allow formation of ^{125}I-labelled TPO/TPO autoantibody complexes in the liquid phase. The labelled complexes are then precipitated by addition of solid phase protein A. The amount of TPO Abs in the test serum is a function of the ^{125}I-labelled antigen precipitated and the results are read off a standard curve.

TPO labelled indirectly (for example linked to a radioactively labelled or chemically labelled or enzyme labelled antibody) can also be used in assay systems to measure TPO Abs.

The modified TPO may be labelled with, for example, ^{35}S (for example, as ^{35}S-methionine), typically carried out using a methionine-free rabbit reticulocyte lysate system. Unreacted ^{35}S-methionine may be removed, for example, by passing through a Sephadex G50 column. Alternatively, other radioisotope labels may be used; as further alternatives enzyme labels or luminescent or fluorescent labels or dyes may be used.

Preferably, the modified TPO genes are truncated and/or deleted by digestion at restriction enzyme sites and isolation of the resulting modified genes (typically on agarose gels).

In one example, the modified TPO genes containing truncations and/or deletions may be cloned into a pTZ18 vector under the control of a T7 promoter. Other suitable vectors and/or promotors may be used if appropriate.

In one embodiment, a Promega TnT transcription/translation kit may be used during the production and/or labelling of the modified TPO. Other suitable protein expression systems may be used if appropriate.

The invention may be more clearly understood by the following description of the accompanying figures, which is given by way of example only.

In the drawings, Figure 1 shows an outline of restriction enzyme sites (frequency and position in the DNA sequence) used to introduce modifications into the TPO sequence;

Figure 2 shows the TPO DNA sequence/translated protein sequence (with selected restriction enzyme sites shown);

Figure 3 is a schematic representation of modifications introduced into the TPO sequence;

Figure 4a is a schematic map of an unmodified pTZ18 cloning vector suitable for use according to the invention;

Figure 4b is a pTZ18 vector DNA sequence and Linker sequence suitable for use according to the invention;

Figure 5 shows a T7 promoter sequence suitable for use according to the invention; and

Figure 6 is a summary of the reactivity of modified TPO with Abs in sera from patients with AITD, Addison's disease and healthy blood donors.

Sera from 20 patients with AITD (16 Graves' and 4 Hashimoto's), 8 patients with Addison's disease and 9 healthy blood donors were studied for reactivity with TPO Abs using an immunoprecipitation assay. In the assay, sera were incubated with the ^{35}S labelled variously modified TPO preparations for 2 hours at room temperature followed by incubation for 1 hour with solid phase protein A to bind ^{35}S modified TPO-TPO autoantibody complexes. The protein A-bound complexes were then counted for ^{35}S and results computed as percentage binding of total radioactivity applied. In addition, results are given as a percentage of binding of the ^{35}S-labelled 90kD TPO reference protein.

Modifications to the TPO gene were carried out by restriction enzyme digestion at specific enzyme sites in the TPO sequence (see Figures 1 to 3). These truncated and/or deleted TPO gene sequences were then isolated and purified using a commercial GENECLEAN kit (GENECLEAN II, ANACHEM, Luton, Beds). Briefly, the digested TPO gene fragments were run on ethidium bromide stained agarose gels, and the relevant DNA bands excised from the gel using a scalpel blade and placed in a microfuge tube.

The approximate volume of the gel slice was then estimated by weight and 3 volumes of 6M sodium iodide stock solution added. The agarose slice was then dissolved by placing the microfuge tube at 55° C for 5 minutes. 10μl of the kit GLASSMILK (insoluble silica matrix stock) was added to the dissolved agarose solution, mixed and the suspension incubated for 5 minutes on ice. The GLASSMILK/DNA complex was then pelleted in a microfuge for 5 seconds. This pellet was then washed 3 times with GENECLEAN II NEW WASH solution before being eluted from the GLASSMILK matrix with sterile water at 55°C for 3 minutes.

The purified TPO gene fragments cloned into the modified pTZ18 vector (Figures 4a and 4b), containing a unique linker, (Figure 4b) under the control of the T7 promoter (see Figure 5) were then expressed and labelled with ^{35}S-methionine in an *in vitro* rabbit reticulocyte lystate system using a Promega TnT transcription/translation kit. The reactions were carried out with a methionine-free rabbit reticulocyte lysate in the presence of ^{35}S-methionine (10Ci/L; Amersham). The reaction mixture was then passed through a 0.5 x 16cm column of Sephadex G50 (Pharmacia), eluted with 150mmol/L Tris buffer pH8.3,

containing (per litre) 200mmol of NaCl, 10mL of Tween-20 and 10g of bovine serum albumin (Tris buffer); 1mL fractions were collected. The fractions collected were tested for reactivity with TPO Abs by immunoprecipitation assay (IPA), diluted to give about 30,000 counts/min per 50μL and stored in aliquots at - 70°C. Synthesis of proteins coded for by the various constructs was assessed by analysis on acrylamide gels (9%) in SDS followed by autoradiography.

The IPA was carried out with a 96-well filtration plate system incorporating 0.45μm (pore size) filters (Multiscreen, Millipore UK Limited). Aliquots of 50μL of ^{35}S-TPO were incubated with aliquots of diluted serum (50μL diluted 10 fold in Tris buffer) for 2 hours at room temperature with shaking. To each microtiter well was then added 50μL of Protein A-Sepharose (Pharmacia), diluted 10 fold in Tris buffer and the samples were incubated for 1 hour more at room temperature with shaking. The plate was then placed on the suction unit (Millipore UK) and the immune complexes bound to Protein A-Sepharose were washed three times with 200μL of Tris buffer. After this washing, the bottom of each well was punched out into vials containing 1ml of scintillation liquid (Ultima Gold, Packard) and the radioactivity counted in a scintillation counter. Negative pool serum obtained from 10 healthy blood donors was included in each assay as well as rabbit TPO Abs (RSR Limited) as a control. Each experiment was carried out in duplicate and binding to each modified TPO protein was carried out in two separate experiments.

Results were expressed as percent binding of total radioactivity added. In addition, results were expressed as percent binding to 90kDa TPO reference protein.

Figure 3 is a schematic representation of modifications introduced into the TPO sequence. 90kD reference TPO protein was produced by digestion at AccI site. C-terminal truncations were at SacI (AA772-838 deleted); at ClaI (AA631-838 deleted); at SmaI (AA 515-838 deleted) and BamHI (AA455-838 deleted). Internal deletions were: XmaI/SacI (AA 514-772 deleted); ApaI/ApaI (AA 386-652 deleted); NarI/XmaI (AA166-517 deleted); Eco47III/MluI (AA3-324 deleted) and Eco47III/NarI (AA 3-166 deleted). A summary of the modifications introduced into the TPO sequence is given in the following Table 1.

TABLE 1

Summary of Modifications Introduced into the TPO Sequence

Enzyme sites used	Amino acids deleted	Region of deletion
AccI (reference)	838-933	C- terminal
Eco47III/ NarI	3-166	N- terminal
Eco47III/MluI	3-324	N- terminal
NarI/XmaI	166-517	Internal
ApaI/ApaI	386-652	Internal
XmaI/SacI	514-772	Internal
BamHI	455-838	C- terminal
SmaI	515-838	C- terminal
ClaI	631-838	C- terminal
SacI	772-838	C- terminal

Results of TPO autoantibody binding are shown in the following Tables 2 and 3:

Table 2 shows results of TPO autoantibody binding to 90kD reference and modified TPO in AITD, Addison's disease and healthy blood donors. Results are expressed as % binding of total radioactivity added and are mean±SD of two separate experiments.

TABLE 2

Group	AA 838-933	AA772-838	AA631-838	AA515-838	AA455-838	AA514-772	AA386-652	AA166-517	AA3-324	AA3-166
AITD n=20	18.7% +/- 5.3%	15.7% +/- 4.7%	2.2% +/- 1.0%	2.8% +/- 1.3%	3.4% +/- 1.1%	2.7% +/- 1.3%	2.7% +/- 2.3%	6.7% +/- 3.4%	10.7% +/- 2.7%	14.8% +/- 3.0%
Addisons's n=8	10.0% +/- 3.9%	10.3% +/- 3.9%	2.8% +/- 2.1%	1.6% +/- 0.3%	1.9% +/- 0.5%	2.1% +/- 0.4%	2.2% +/- 1.7%	4.5% +/- 4.2%	7.5% +/- 2.4%	11.4% +/- 3.3%
Normals n=9	6.0% +/- 2.2%	6.6% +/- 2.3%	1.3% +/- 0.14%	1.5% +/- 0.2%	1.9% +/- 0.3%	1.6% +/- 0.2%	1.6% +/- 0.3%	2.6% +/- 2.3%	6.5% +/- 2.5%	9.4% +/- 2.7%

Table 3 shows TPO autoantibody binding to 90kD reference and modified TPO in AITD, Addison's disease and healthy blood donors. Results are expressed as % binding to 90kD TPO reference protein and are mean ±SD of two separate experiments.

TABLE 3

Group	AA772-838 SacI	AA515-838 SmaI	AA455-838 BamHI	AA631-838 ClaI	AA386-652 ApaI/ApaI	AA166-517 NarI/XmaI	AA514-772 SacI/XmaI	AA3-166 Eco47III/NarI	AA3-324 Eco47III/MluI
AITD n=20	84% +/- 5%	15% +/- 5%	19% +/- 5%	15% +/- 4%	15% +/- 8%	36% +/- 11%	15% +/- 5%	81% +/- 9%	58% +/- 5%
Addisons's n=8	104% +/- 7%	18% +/- 6%	22% +/- 8%	27% +/- 11%	23% +/- 17%	38% +/- 22%	23% +/- 6%	123% +/- 30%	80% +/- 20%
Normals n=9	110% +/- 8%	28% +/- 10%	35% +/- 13%	24% +/- 8%	30% +/- 10%	46% +/- 24%	29% +/- 9%	160% +/- 20%	108% +/- 14%

Figure 6 is a summary of the reactivity of modified TPO with Abs in sera from patients with AITD, Addison's disease and healthy blood donors.

The results show that:

1. Removal of the last 66 amino acids (AA) from the 90kD fragment (truncation at AA 772) had a small (about 15% reduction) effect on TPO autoantibody binding whereas truncations of AA 631-838, 515-838 or 455-838 caused a marked reduction (mean about 80%) in autoantibody binding. Deletion of AA 514-772 and AA 386-652 also had marked effect on autoantibody binding (reduction about 70%). Deletions of AA 166-517 reduced binding by about 60%. The effect on TPO autoantibody binding in sera from patients with AITD, Addison's disease and healthy blood donors were similar.
2. Deletions within the N-terminal part of the protein AA3-166 and AA 3-324 showed an effect on TPO autoantibody binding in sera from patients with AITD (20 and 40% reduction respectively), had small (about 20% for AA 3-324 deletion) or no effect (AA3-166 deletion) on TPO autoantibody binding in Addison's disease but had no effect on TPO autoantibody binding in sera from healthy blood donors.

These studies suggest that TPO Abs from healthy blood donors show different epitope recognition in comparison to TPO Abs from patients with AITD or other autoimmune diseases such as Addison's disease. Other examples of autoimmune diseases include insulin dependent diabetes mellitus, rheumatoid arthritis, systemic lupus erythematosus (SLE), and myaesthenia gravis. These observations may allow differentiation between disease related and unrelated TPO Abs.

CLAIMS:

1. A method of monitoring the reactivity of thyroid peroxidase (TPO) autoantibodies, which comprises:
   (a) labelling modified TPO prepared using expressed modified TPO genes containing truncations and/or deletions; and
   (b) monitoring reactivity of said labelled modified TPO with TPO autoantibodies present in a body fluid from a patient.

2. A method according to claim 1, wherein step (b) is carried out using an immunoprecipitation assay.

3. A method according to claim 1,wherein step (b) is carried out using an enzyme linked immunosorbent assay (ELISA).

4. A method according to claim 1, wherein step (b) is carried out using a chemiluminescence assay.

5. A method according to claim 1, wherein step (b) is carried out using an immuno chromatographic assay.

6. A method according to any of claims 1 to 5, wherein said truncations and/or deletions are produced by digestions at restriction enzyme sites and isolation of the resulting modified genes.

7. A method according to any of claims 1 to 6, wherein a 90kD reference TPO protein is produced by digestion at AccI site.

8. A method according to any of claims 1 to 7, wherein said modified TPO has C-terminal truncations at SacI resulting in deletion of amino acid (AA)772-838; at ClaI resulting in deletion of AA631-838; at SmaI resulting in deletion of AA515-838 and at BamHI resulting in deletion of AA455-838.

9. A method according to any of claims 1 to 8, wherein said modified TPO has internal deletions at XmaI/SacI (wherein AA514-772 are deleted); ApaI/ApaI (wherein AA386-652 are deleted); NarI/XmaI (wherein AA166-517 are deleted); Eco 47III/MluI (wherein AA3-324 are deleted) and Eco47III/NarI (wherein AA3-166 are deleted).

10. A method according to any of claims 1 to 9, wherein said reactivity is monitored in test samples of said body fluid taken from patients having autoimmune disease and control samples of said body fluid from healthy donors, wherein said reactivity of said modified TPO prepared using said genes containing deletions with said test samples results in lowered relative TPO autoantibody binding compared to said reactivity with said control samples.

11. A method according to claim 10, wherein said TPO autoantibody binding is in said test samples having modified TPO with deletions within an N-terminal part of AA3-324 said samples being from patients with autoimmune thyroid disease (AITD) or other autoimmune disease.

12. A method according to any of claims 1 to 10, wherein said TPO autoantibody binding is in said test samples having modified TPO with deletions within an N-terminal part of AA3-166, being from patients with AITD or other autoimmune disease.

13. A method according to any of claims 1 to 9, wherein said reactivity is monitored in test samples of said body fluid taken from patients being screened for autoimmune disease and from control samples of said body fluid from healthy donors, wherein lowered relative TPO autoantibody binding in said test samples relative to said control samples is indicative of suspected autoimmune disease.

14. A method according to any of claims 1 to 13, wherein the difference in the ability of TPO Abs to react with a 90kD reference TPO and modified TPO indicates the presence of disease related or disease unrelated TPO Abs.

15. A method according to any of claims 1 to 14, for the diagnosis of AITD or other autoimmune disease.

16. A method according to claim 15, wherein said AITD is Graves' disease or Hashimoto's thyroiditis.

17. A method according to any of claims 1 to 16, wherein said labelling is carried out with a radioisotope (such as ^{35}S or ^{125}I).

18. A method according to any of claims 1 to 16, wherein said labelling is carried out with an enzyme label (such as horseradish peroxidase or alkaline phosphatase).

19. A method according to any of claims 1 to 16, wherein said labelling is carried out with latex or gold particles, or a chromophore (such as a streptavidin-dye complex).

20. A method according to any of claims 1 to 16, wherein said labelling is carried out with a fluorescent or chemiluminescent reagent.

21. A method according to any of claims 1 to 16, wherein said labelling is carried out with a metal chelate.

22. A method according to any of claims 1 to 16, wherein said labelling is carried out with a bioluminescent compound.

23. A method according to any of claims 1 to 22, wherein said body fluid comprises whole blood.

24. A method according to any of claims 1 to 22, wherein said body fluid comprises serum or plasma.

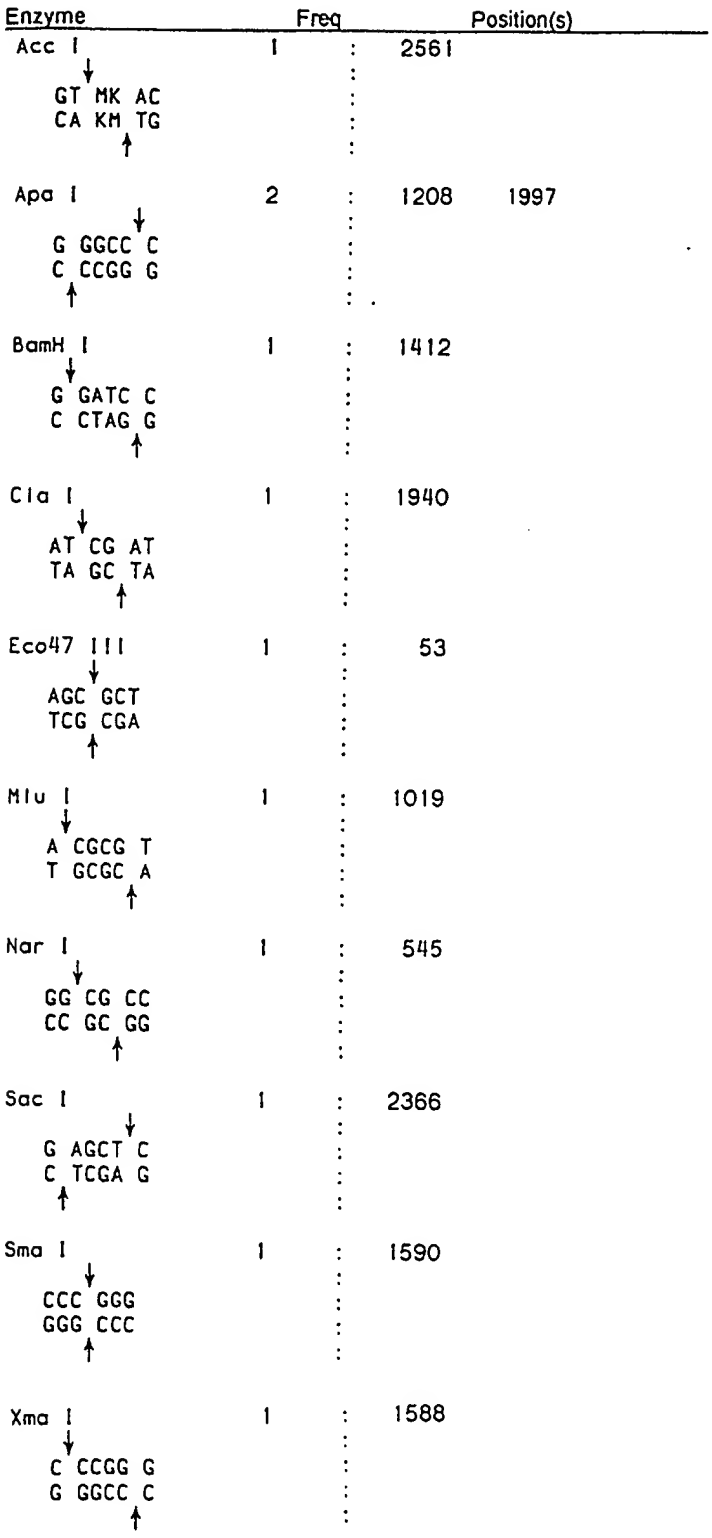
25. A method according to any of claims 1 to 22, wherein said body fluid comprises amniotic fluid.

26. A method according to any of claims 1 to 25, wherein said modified TPO has an N-terminal deletion between AA3-166 of TPO.

27. A method according to any of claims 1 to 25, wherein said modified TPO has an N-terminal deletion between AA3-324 of TPO.

28. A method according to any of claims 1 to 27, for use in the differentiation between disease-related TPO Abs and disease unrelated TPO Abs.

Figure 1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Freq
Position(s)
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CA KM TG
Apa I
2
1208
1997
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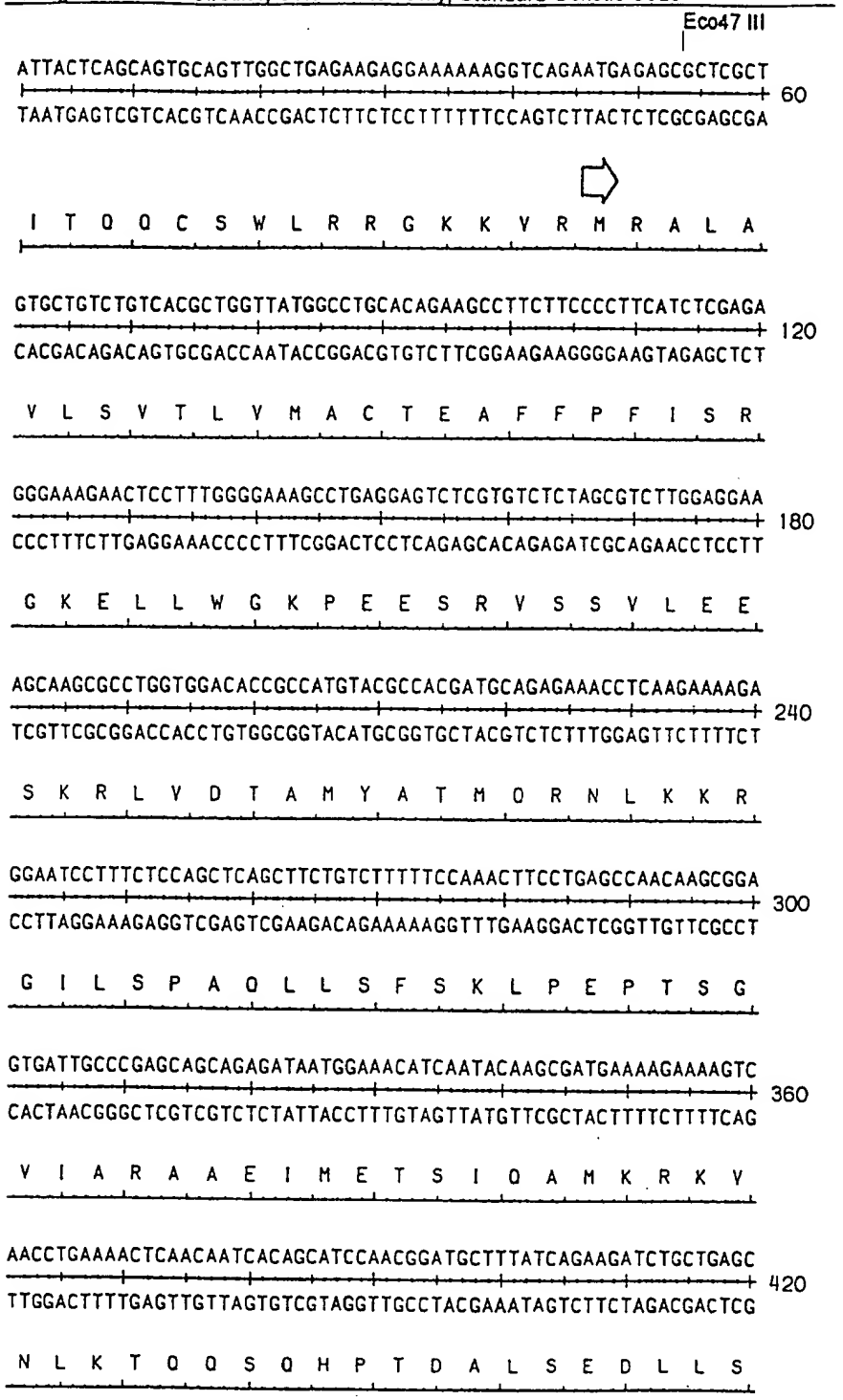
C CCGG G
BamH I
1
1412
G GATC C
C CTAG G
Cla I
1
1940
AT CG AT
TA GC TA
Eco47 III
1
53
AGC GCT
TCG CGA
Mlu I
1
1019
A CGCG T
T GCGC A
Nar I
1
545
GG CG CC
CC GC GG
Sac I
1
2366
G AGCT C
C TCGA G
Sma I
1
1590
CCC GGG
GGG CCC
Xma I
1
1588
C CCGG G
G GGCC C

Figure 2

TPO.MPD (1 > 2847) Site and Sequence
Enzymes : 10 of 445 enzymes (Filtered)
Settings : Circular, Certain Sites Only, Standard Genetic Code



TPO.MPD (1 > 2847) Site and Sequence

```
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 I  I  A  N  M  S  G  C  L  P  Y  M  L  P  P  K  C  P  N  T

TGCCTGGCGAACAAATACAGGCCCATCACAGGAGCTTGCAACAACAGAGACCACCCCAGA
ACGGACCGCTTGTTTATGTCCGGGTAGTGTCCTCGAACGTTGTTGTCTCTGGTGGGGTCT

 C  L  A  N  K  Y  R  P  I  T  G  A  C  N  N  R  D  H  P  R

      Nar I
      |
TGGGGCGCCTCCAACACGGCCCTGGCACGATGGCTCCCTCCAGTCTATGAGGACGGCTTC
ACCCCGCGGAGGTTGTGCCGGGACCGTGCTACCGAGGGAGGTCAGATACTCCTGCCGAAG

 W  G  A  S  N  T  A  L  A  R  W  L  P  P  V  Y  E  D  G  F

AGTCAGCCCCGAGGCTGGAACCCCGGCTTCTTGTACAACGGGTTCCCACTGCCCCCGGTC
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 S  Q  P  R  G  W  N  P  G  F  L  Y  N  G  F  P  L  P  P  V

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GCCCTCCACTGTTCTGTACAGTAAGTTCAAAGTTTACTCCAACAGTGTCTACTACTGGCG

 R  E  V  T  R  H  V  I  Q  V  S  N  E  V  V  T  D  D  D  R

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 Y  S  D  L  L  M  A  W  G  Q  Y  I  D  H  D  I  A  F  T  P

CAGAGCACCAGCAAAGCTGCCTTCGGGGGAGGGGCTGACTGCCAGATGACTTGTGAGAAC
GTCTCGTGGTCGTTTCGACGGAAGCCCCCTCCCCGACTGACGGTCTACTGAACACTCTTG

 Q  S  T  S  K  A  A  F  G  G  G  A  D  C  Q  M  T  C  E  N
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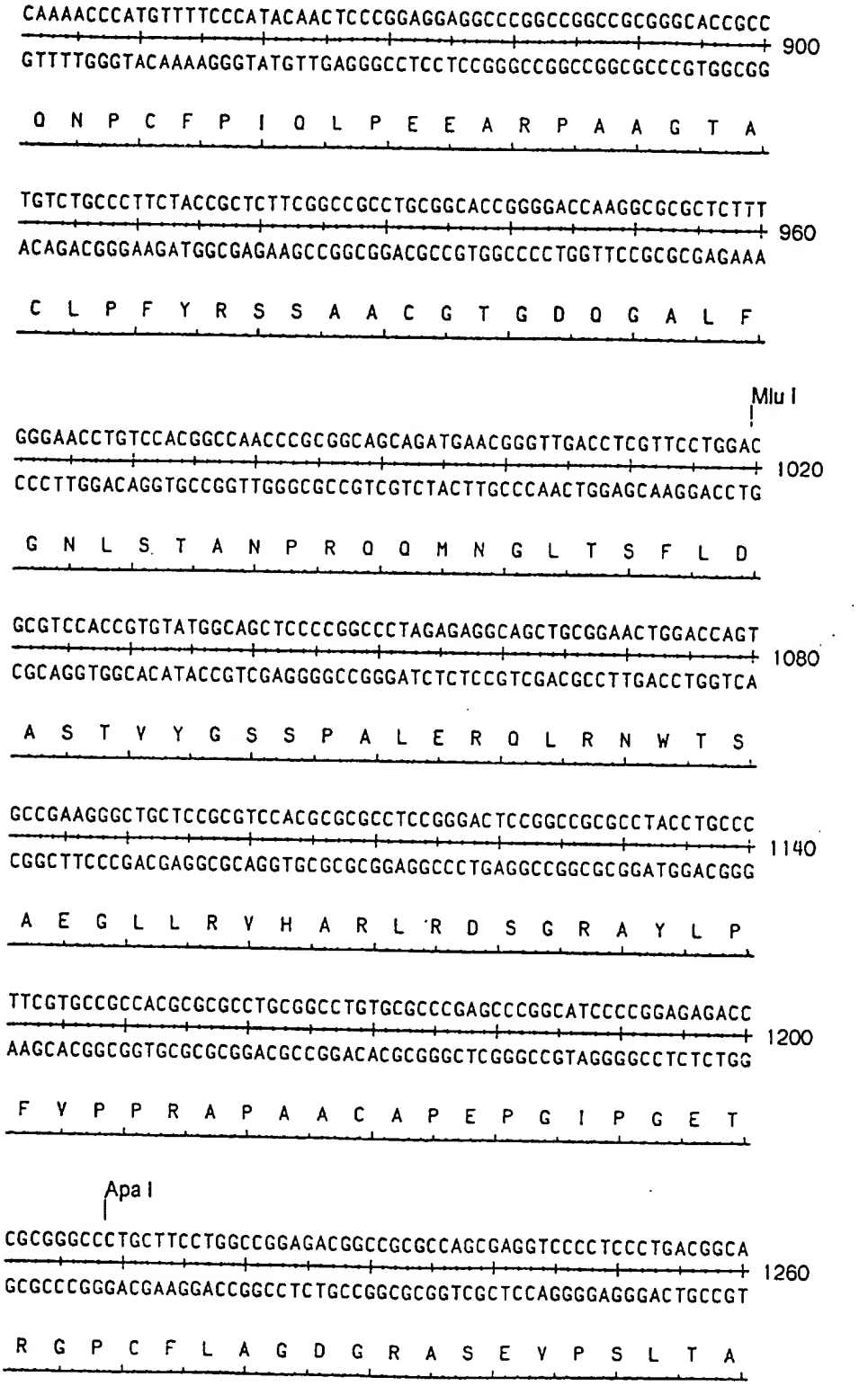
TPO.MPD (1 > 2847) Site and Sequence



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 L  H  T  L  W  L  R  E  H  N  R  L  A  A  A  L  K  A  L  N

GCGCACTGGAGCGCGGACGCCGTGTACCAGGAGGCGCGCAAGGTCGTGGGCGCTCTGCAC
                                                              1380
CGCGTGACCTCGCGCCTGCGGCACATGGTCCTCCGCGCGTTCCAGCACCCGCGAGACGTG

 A  H  W  S  A  D  A  V  Y  Q  E  A  R  K  V  V  G  A  L  H

                          BamH I
                          |
CAGATCATCACCCTGAGGGATTACATCCCCAGGATCCTGGGACCCGAGGCCTTCCAGCAG
                                                              1440
GTCTAGTAGTGGGACTCCCTAATGTAGGGGTCCTAGGACCCTGGGCTCCGGAAGGTCGTC

 Q  I  I  T  L  R  D  Y  I  P  R  I  L  G  P  E  A  F  Q  Q

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                                                              1500
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 Y  V  G  P  Y  E  G  Y  D  S  T  A  N  P  T  V  S  N  V  F

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                                                              1560
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 S  T  A  A  F  R  F  G  H  A  T  I  H  P  L  V  R  R  L  D

                    Xma I
                    |
                    | Sma I
                    | |
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                                                              1620
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                                                              1680
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TPO.MPD (1 > 2847) Site and Sequence

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CACGACAGGTTAAGGTCGTGGAACTTAGACCGCAGGTAGTTGGACGTCTCCCCGGCCCTG 1800

 V  L  S  N  S  S  T  L  N  L  A  S  I  N  L  Q  R  G  R  D

CACGGGCTGCCAGGTTACAATGAGTGGAGGGAGTTCTGCGGCCTGCCTCGCCTGGAGACC
GTGCCCGACGGTCCAATGTTACTCACCTCCCTCAAGACGCCGGACGGAGCGGACCTCTGG 1860

 H  G  L  P  G  Y  N  E  W  R  E  F  C  G  L  P  R  L  E  T

CCCGCTGACCTGAGCACAGCCATCGCCAGCAGGAGCGTGGCCGACAAGATCCTGGACTTG
GGGCGACTGGACTCGTGTCGGTAGCGGTCGTCCTCGCACCGGCTGTTCTAGGACCTGAAC 1920

 P  A  D  L  S  T  A  I  A  S  R  S  V  A  D  K  I  L  D  L

                    Cla I
                      |
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 Y  K  H  P  D  N  I  D  V  W  L  G  G  L  A  E  N  F  L  P

                 Apa I
                   |
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TCCCGAGCCTGTCCCGGGGACAAACGGACAGAGTAACCCTTCGTCTACTTCCGAGACGCC 2040

 R  A  R  T  G  P  L  F  A  C  L  I  G  K  Q  M  K  A  L  R

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CTACCACTGACCAAAACCACCCTCTTGTCGGTGCAGAAGTGCCTACGTGTCTCCGCACTC 2100

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TPO.MPD (1 > 2847) Site and Sequence

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TACCTACGGAAGGTTCAGCCGTTTAAGGGACTTCTGAAACTCAGAACACTGTCGTAGGGA 2220

 M  D  A  F  Q  V  G  K  F  P  E  D  F  E  S  C  D  S  I  P

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 G  M  N  L  E  A  W  R  E  T  F  P  Q  D  D  K  C  G  F  P

GAGAGCATGGAGAATGGGGACTTTGTGCACTGTGAGGAGTCTGGGAGGCGCGTGCTGGTG
CTCTCGTACCTCTTACCCCTGAAACACGTGACACTCCTCAGACCCTCCGCGCACGACCAC 2340

 E  S  M  E  N  G  D  F  V  H  C  E  E  S  G  R  R  V  L  V

                    Sac I
                    |
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ATAAGGACGGCCGTGCCCATACTCGAGGTTCCGGCCCTCGTCGAGTGAACGTGGGTCCTT 2400

 Y  S  C  R  H  G  Y  E  L  Q  G  R  E  Q  L  T  C  T  Q  E

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CCTACCCTAAAGGTCGGAGGGGAGACGTTTCTACACTTGCTCACACGTCTGCCACGGGTG 2460

 G  W  D  F  Q  P  P  L  C  K  D  V  N  E  C  A  D  G  A  H

CCCCCCTGCCACGCCTCTGCGAGGTGCAGAAACACCAAAGGCGGCTTCCAGTGTCTCTGC
GGGGGGACGGTGCGGAGACGCTCCACGTCTTTGTGGTTTCCGCCGAAGGTCACAGAGACG 2520

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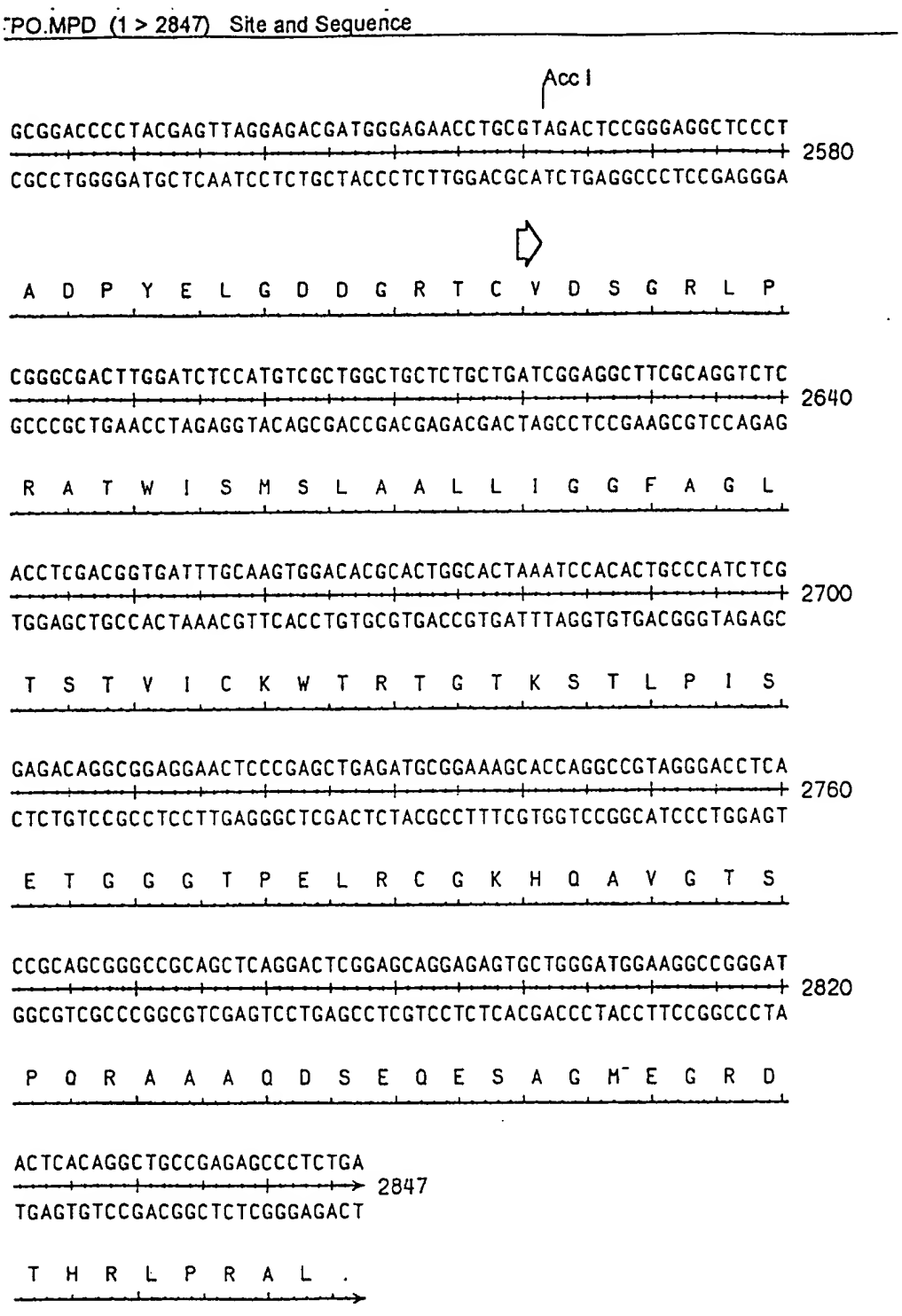
TPO.MPD (1 > 2847) Site and Sequence
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A D P Y E L G D D G R T C V D S G R L P
CGGGCGACTTGGATCTCCATGTCGCTGGCTGCTCTGCTGATCGGAGGCTTCGCAGGTCTC
2640
GCCCGCTGAACCTAGAGGTACAGCGACCGACGAGACGACTAGCCTCCGAAGCGTCCAGAG
R A T W I S M S L A A L L I G G F A G L
ACCTCGACGGTGATTTGCAAGTGGACACGCACTGGCACTAAATCCACACTGCCCATCTCG
2700
TGGAGCTGCCACTAAACGTTCACCTGTGCGTGACCGTGATTTAGGTGTGACGGGTAGAGC
T S T V I C K W T R T G T K S T L P I S
GAGACAGGCGGAGGAACTCCCGAGCTGAGATGCGGAAAGCACCAGGCCGTAGGGACCTCA
2760
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E T G G G T P E L R C G K H Q A V G T S
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2820
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P Q R A A A Q D S E Q E S A G M E G R D
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2847
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T H R L P R A L .

Figure 3

Schematic representation of modifications introduced into the TPO sequence

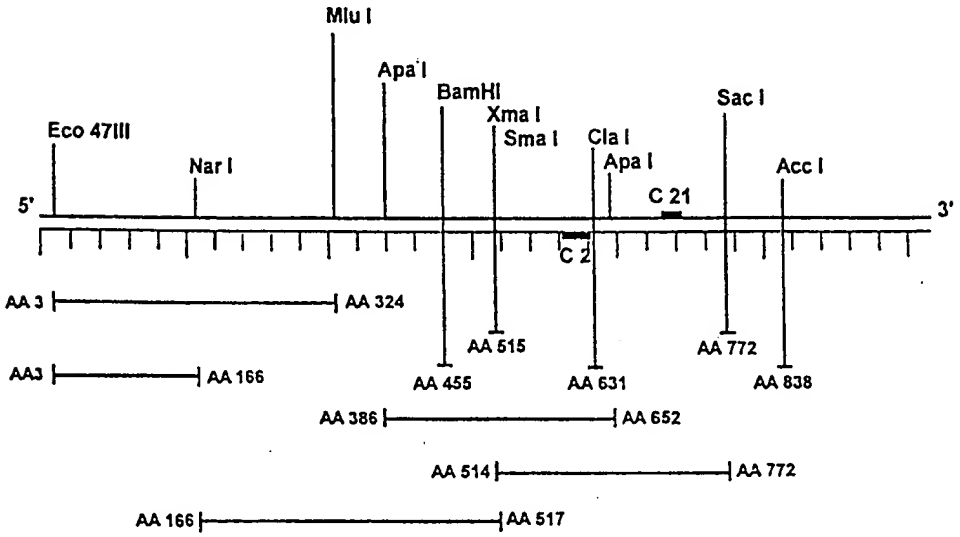
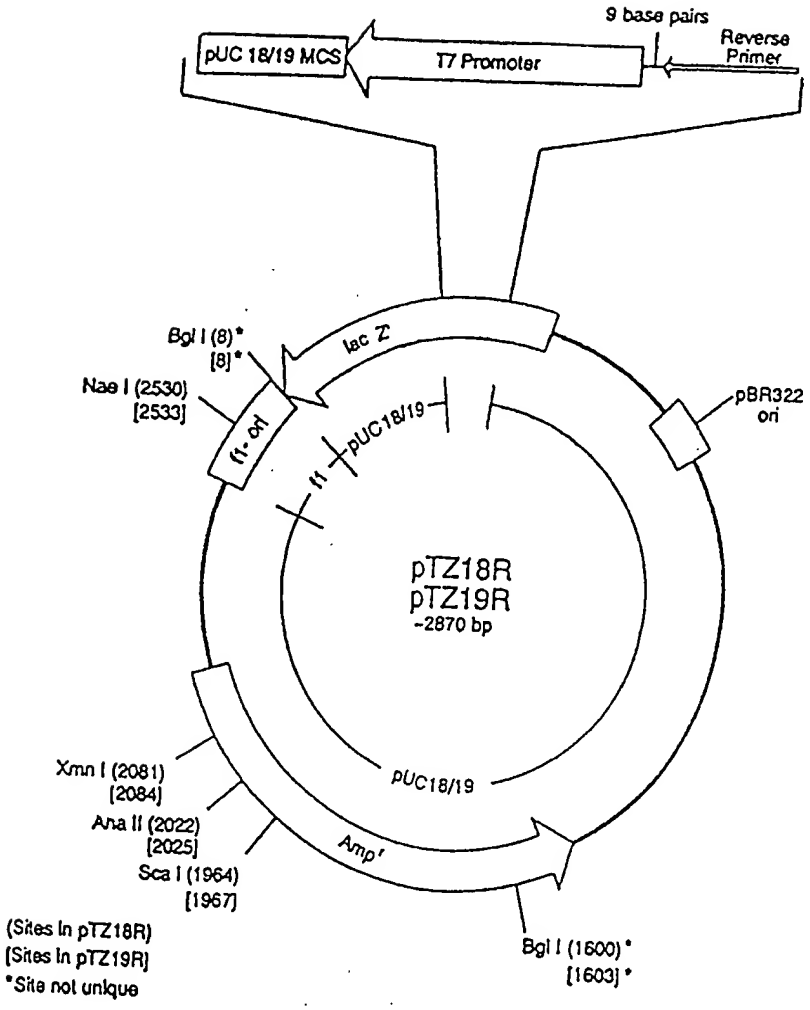


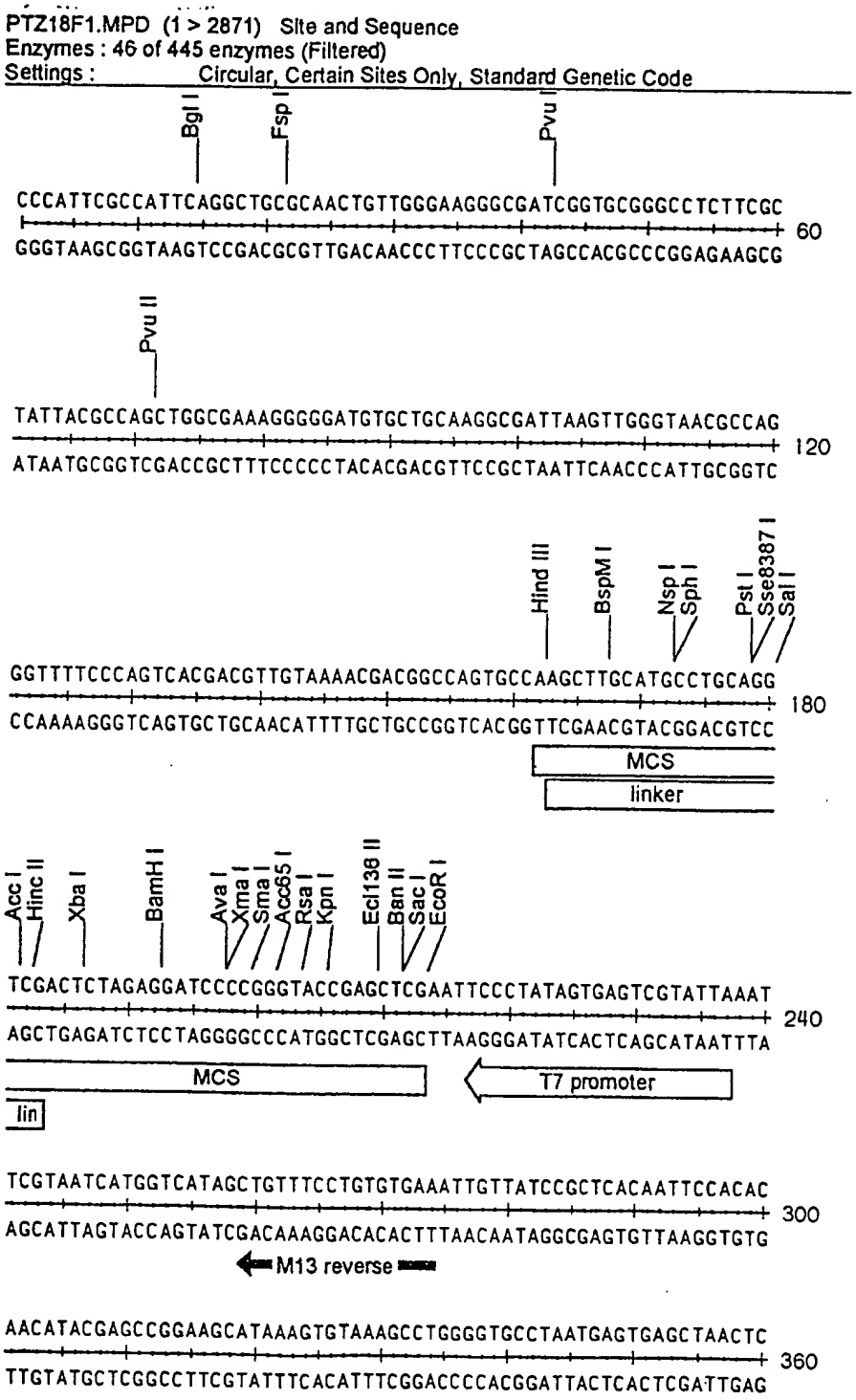
Figure 4a



Schematic map of the pTZ18 cloning vector.

The positions of some restriction enzyme sites are marked. Positions of the ampicillin resistence gene (AMP^r) and the lac Z gene are indicated. Positions of the f1 and pBR322 origin of replication are indicated. The multiple cloning site (MCS) positioned 5′ to the T7 promoter, and the region corresponding to the M13 reverse sequencing primer are indicated.

Figure 4b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60
Pvu II
TATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAG
ATAATGCGGTCGACCGCTTTCCCCCTACACGACGTTCCGCTAATTCAACCCATTGCGGTC
120
Hind III
BspM I
Nsp I
Sph I
Pst I
Sse8387 I
Sal I
GGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAAGCTTGCATGCCTGCAGG
CCAAAAGGGTCAGTGCTGCAACATTTTGCTGCCGGTCACGGTTCGAACGTACGGACGTCC
180
MCS
linker
Acc I
Hinc II
Xba I
BamH I
Ava I
Xma I
Sma I
Acc65 I
Rsa I
Kpn I
Ecl136 II
Ban II
Sac I
EcoR I
TCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCCCTATAGTGAGTCGTATTAAAT
AGCTGAGATCTCCTAGGGGCCCATGGCTCGAGCTTAAGGGATATCACTCAGCATAATTTA
240
MCS
T7 promoter
lin
TCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACAC
AGCATTAGTACCAGTATCGACAAAGGACACACTTTAACAATAGGCGAGTGTTAAGGTGTG
300
M13 reverse
AACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTC
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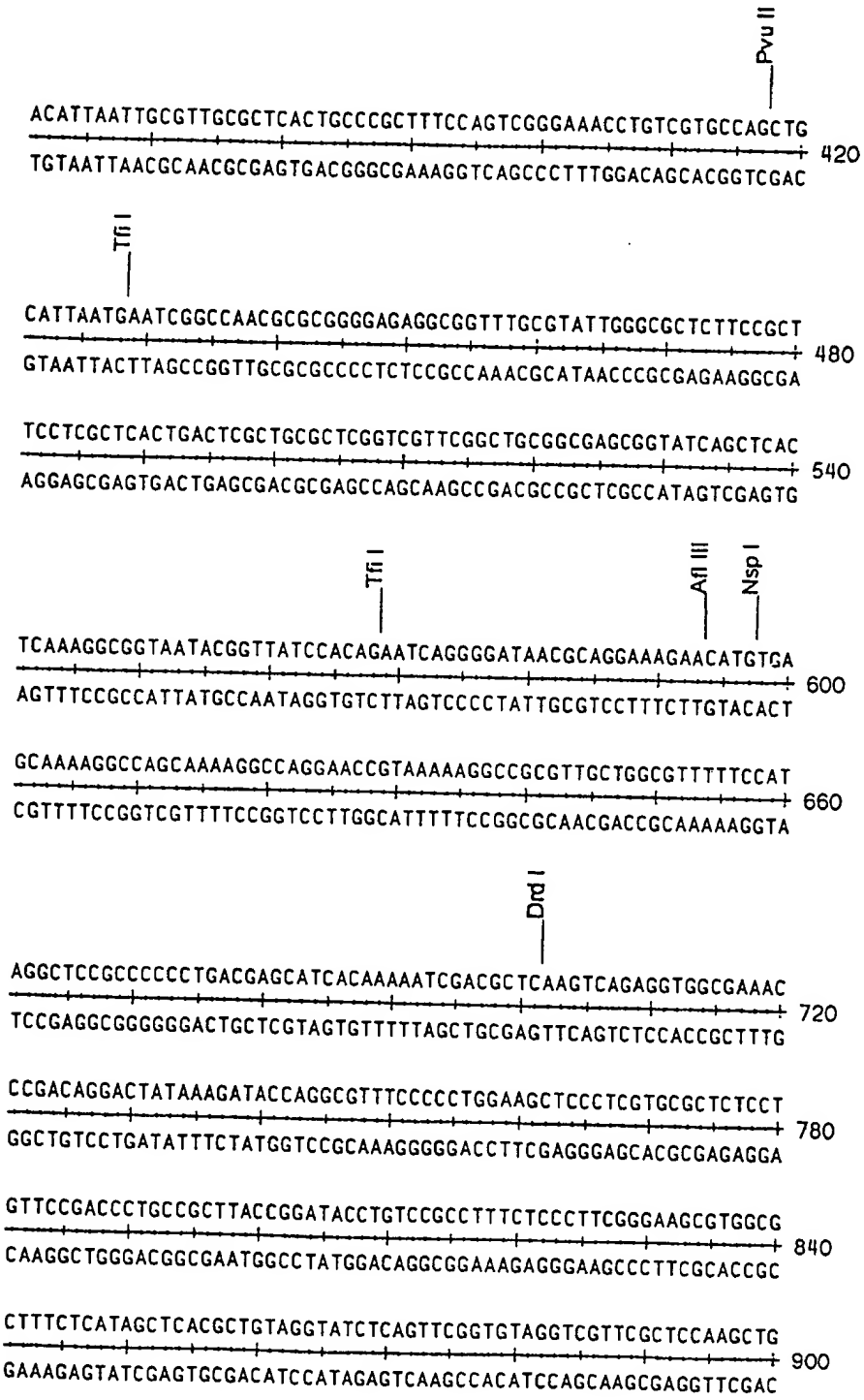
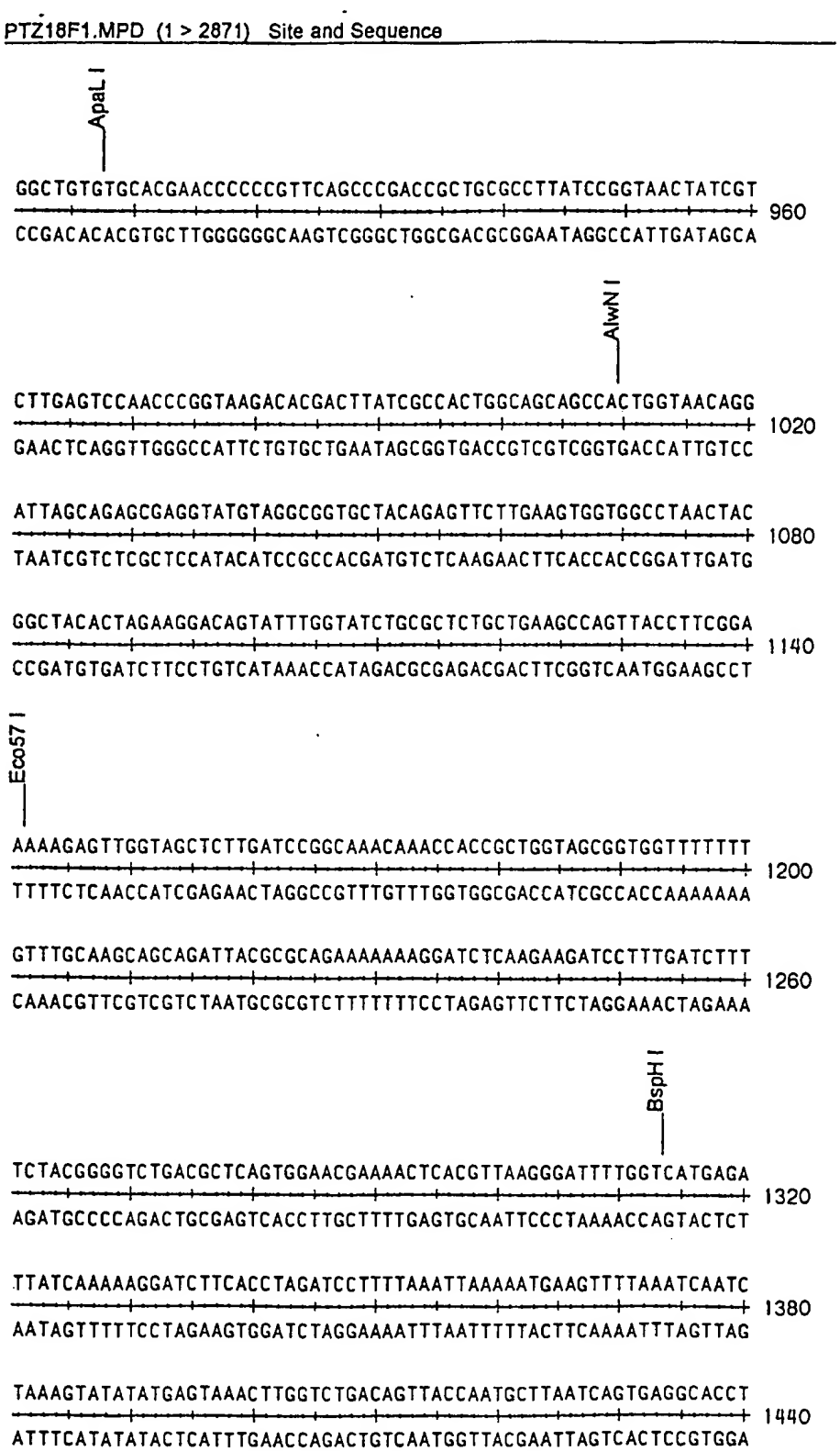
PTZ18F1.MPD (1 > 2871) Site and Sequence

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960
AlwN I
CTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGG
GAACTCAGGTTGGGCCATTCTGTGCTGAATAGCGGTGACCGTCGTCGGTGACCATTGTCC
1020
ATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTAC
TAATCGTCTCGCTCCATACATCCGCCACGATGTCTCAAGAACTTCACCACCGGATTGATG
1080
GGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGA
CCGATGTGATCTTCCTGTCATAAACCATAGACGCGAGACGACTTCGGTCAATGGAAGCCT
1140
Eco57 I
AAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTT
TTTTCTCAACCATCGAGAACTAGGCCGTTTGTTTGGTGGCGACCATCGCCACCAAAAAAA
1200
GTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTT
CAAACGTTCGTCGTCTAATGCGCGTCTTTTTTTCCTAGAGTTCTTCTAGGAAACTAGAAA
1260
BspH I
TCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGA
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1320
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1380
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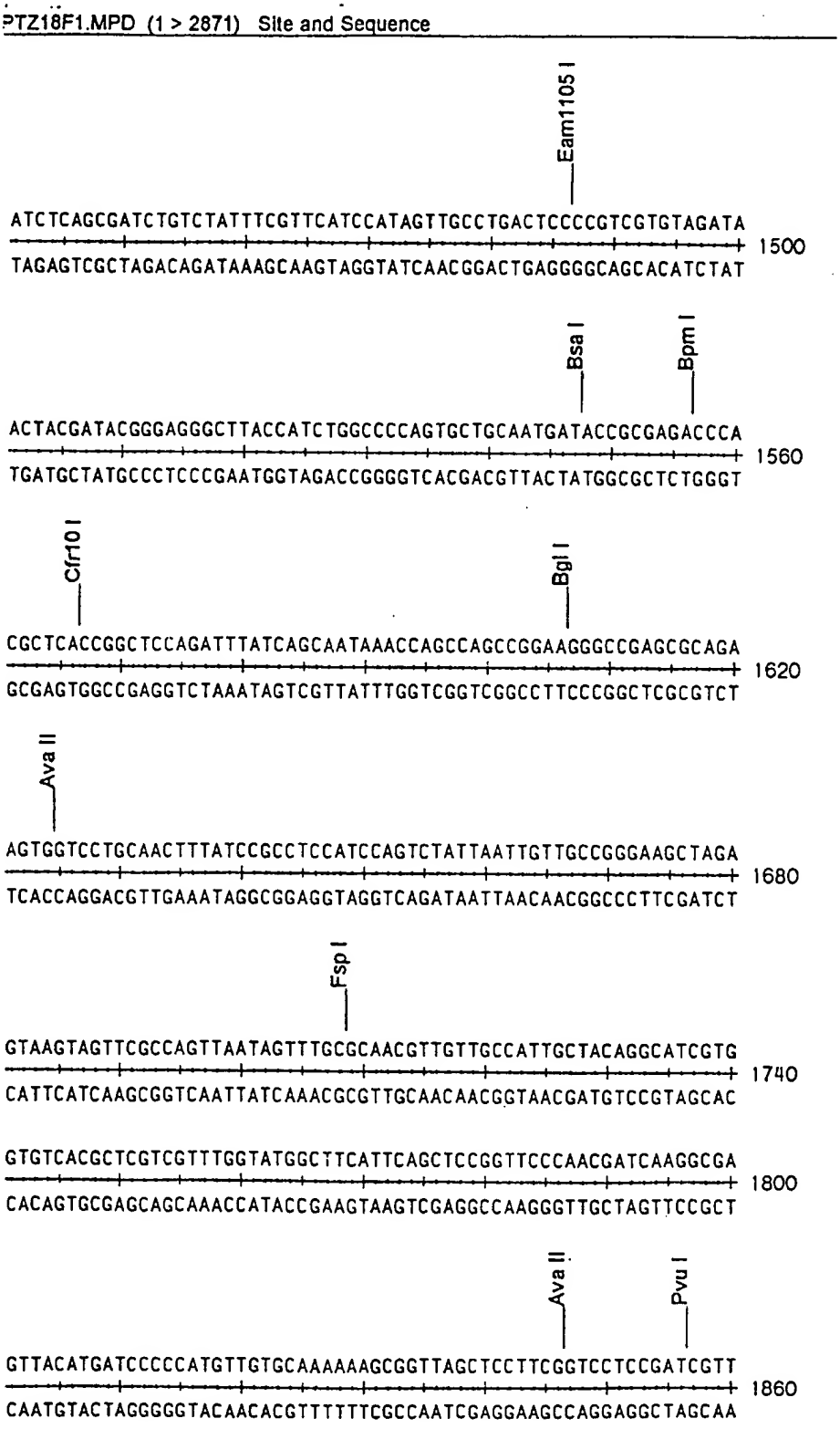
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1500
Bsa I
Bpm I
ACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCA
TGATGCTATGCCCTCCCGAATGGTAGACCGGGGTCACGACGTTACTATGGCGCTCTGGGT
1560
Cfr10 I
Bgl I
CGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGA
GCGAGTGGCCGAGGTCTAAATAGTCGTTATTTGGTCGGTCGGCCTTCCCGGCTCGCGTCT
1620
Ava II
AGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGA
TCACCAGGACGTTGAAATAGGCGGAGGTAGGTCAGATAATTAACAACGGCCCTTCGATCT
1680
Fsp I
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CATTCATCAAGCGGTCAATTATCAAACGCGTTGCAACAACGGTAACGATGTCCGTAGCAC
1740
GTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGA
CACAGTGCGAGCAGCAAACCATACCGAAGTAAGTCGAGGCCAAGGGTTGCTAGTTCCGCT
1800
Ava II
Pvu I
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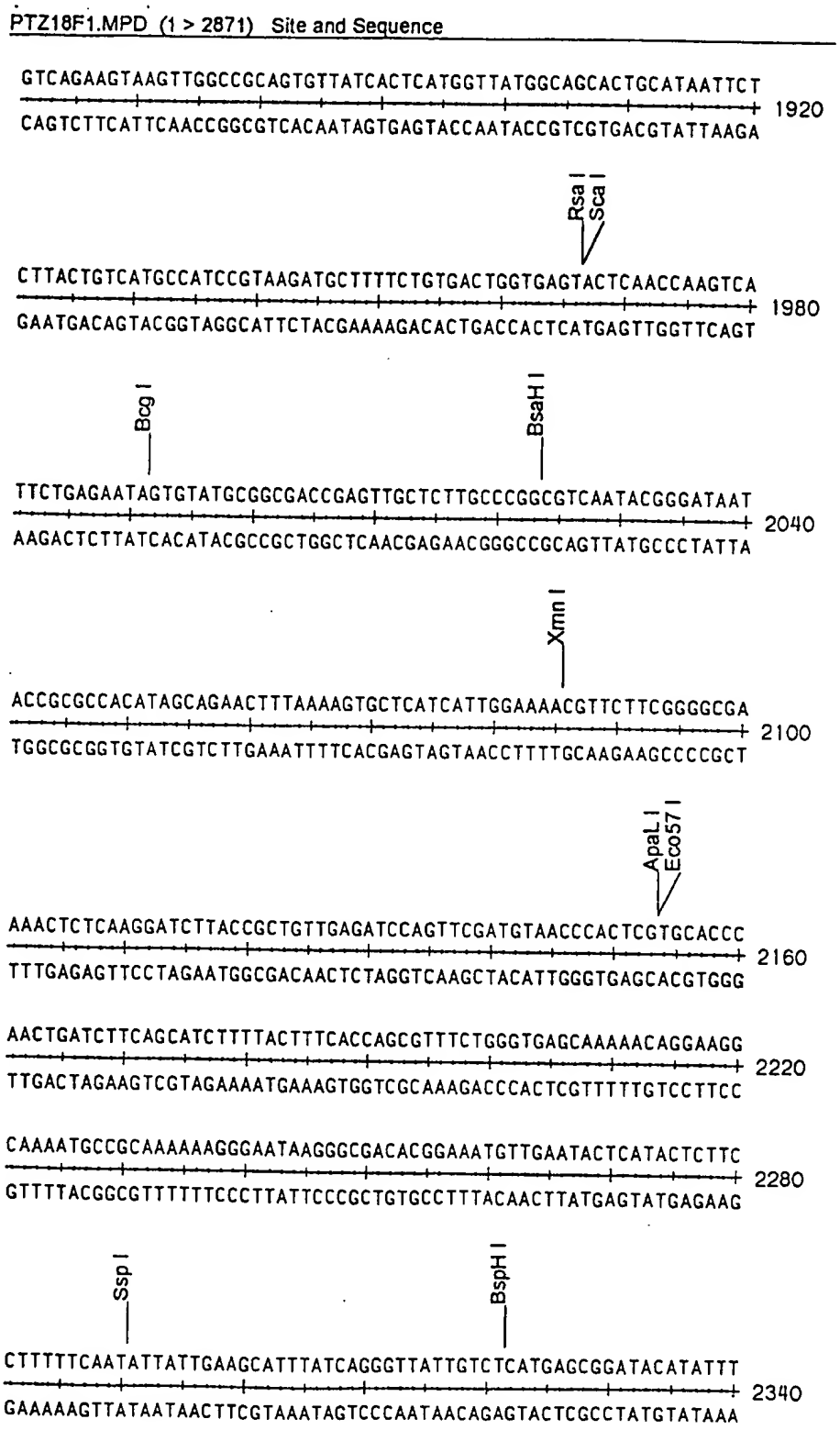
1860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Rsa I
Sca I
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GAATGACAGTACGGTAGGCATTCTACGAAAAGACACTGACCACTCATGAGTTGGTTCAGT
1980
Bcg I
BsaH I
TTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAAT
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2040
Xmn I
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2100
ApaL I
Eco57 I
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2160
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2220
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GTTTTACGGCGTTTTTTCCCTTATTCCCGCTGTGCCTTTACAACTTATGAGTATGAGAAG
2280
Ssp I
BspH I
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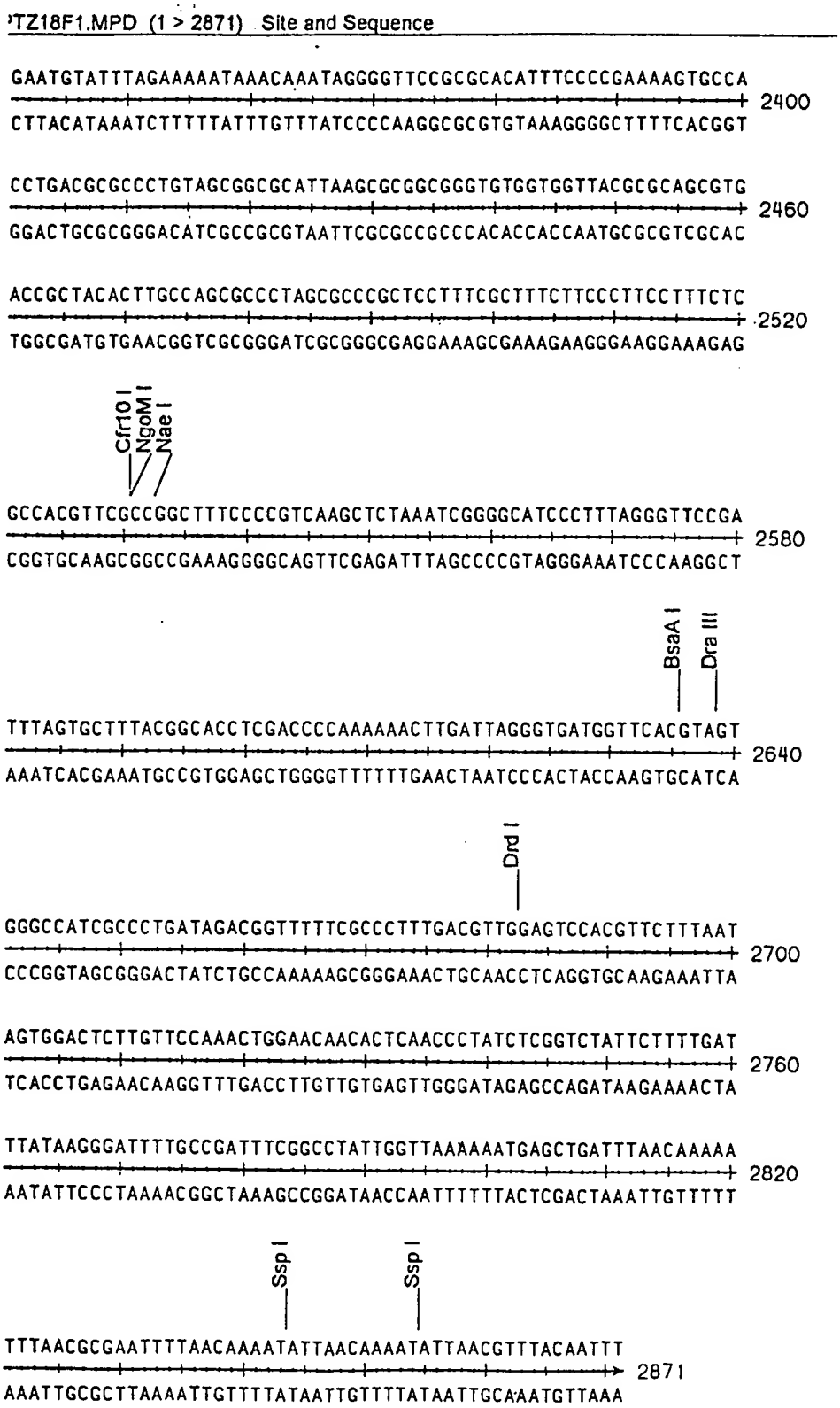
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2460
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Cfr10 I
NgoM I
Nae I
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2640
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Drd I
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2700
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2820
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Ssp I
Ssp I
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2871
AAATTGCGCTTAAAATTGTTTTATAATTGTTTTATAATTGCAAATGTTAAA

Legend to pTZ18 vector sequence

Nucleotide sequence of the plasmid vector sequence pTZ18. The region corresponding to the M13 reverse sequencing primer is indicated (← M13 reverse —). The multiple cloning site is boxed [MCS]. The region corresponding to the T7 promoter is indicated [T7 promoter] The position of the unique pTZ18 linker which replaced part of the MCS is boxed [Linker] sequence of linker is given

Sequence of pTZ18 linker

```
                5'-CGACTGACATCGATCTGACTGACTGA-3'
AccI                :::::::::::::::::::::::::          HindIII
              3'-TGACTGTAGCTAGACTGACTGACTTCGA-5'
```

The pTZ18 cloning vector was modified to include a unique linker which was designed to include a ClaI restriction enzyme site for cloning and a series of TGA stop codons which ensured translation termination of all truncated TPO genes. The modified pTZ18 vector was constructed by cloning the linker into the AccI and HindIII sites of the multiple cloning site of pTZ18 cloning vector.

Figure 5

Sequence of T7 promoter

TAATACGACTCACTATAGGGA

T7 transcription start

Figure 6